J. Helminthol. Soc. Wash.
59(1), 1992, pp. 22–24

Zygocotyle lunata: Laboratory Maintenance in Snails and Mice

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ABSTRACT: A reliable method for laboratory maintenance of all stages of *Zygocotyle lunata* (Digenea) is described which permits further detailed studies of relatively poorly known stages of the life history. The experimental infection of the snail host, *Helisoma anceps,* was achieved by the feeding of incubated eggs of the fluke, rather than exposure to hatched miracidia. Techniques for obtaining maximum numbers of eggs, incubating eggs, and storage of encysted metacercariae also are described.

KEY WORDS: *Zygocotyle lunata, Helisoma anceps,* laboratory infection, Digenea.

The recent monograph of Sey (1991) on the biology of amphistome flukes demonstrates that since the comprehensive investigation of Willey (1941), only a few experimental studies have been published concerning the cecal worm, *Zygocotyle lunata* (Diesing, 1836) Stunkard, 1916. Most of these studies involve aspects of adult development in mammalian and avian hosts (Bacha, 1964; Fried, 1970; Joyner and McDaniel, 1970; Fried and Nelson, 1978; Fried and Gainsburg, 1979; Huffman, et al., 1991). Other studies, such as Fried and Wilson (1981) and Fried et al. (1978) are concerned with the encysted metacercaria. Consequently, there are few data on the free-living and intramolluscan stages of *Z. lunata,* except for the original life history study of Willey (1941).

In studying a population of *Helisoma anceps* infected with *Z. lunata* in 1989, it became apparent why so few recent studies have dealt with pre-adult stages; Willey's (1941) account of the ease with which snails could be infected by miracidia could not be confirmed by the writer, an experience shared by Drs. B. Fried, W. J. Bacha, and J. E. Huffman (pers. comm.). All of their work has originated with field-collected, naturally infected snails.

The present study was undertaken to determine whether snails could be infected in the laboratory, and whether maintenance of *Z. lunata* could be improved to make this trematode a more accessible system for experimental and classroom study.

Materials and Methods

Helisoma anceps, collected in 1989 from 2 ponds adjacent to the 10th and 18th holes of the Greencrest Golf Club, Butler Co., Ohio, were infected with an amphistome cercaria species which rapidly encysted on the surface of bowls in which infected snails were kept. Encysted metacercariae were given to Balb C mice by stomach intubation. Adult worms that developed in the cecum and colon were identified as *Zygocotyle lunata.*

Fingerbowls and petri dishes used to collect encysted metacercariae were stored in aerated tap water in a refrigerator at 8° to 10°C for up to 6 mo. Eggs of *Z. lunata* were obtained by placing gravid adults in 0.95% saline in depression dishes kept at either room temperature (23°–26°) or 37°C for several hours. After rinsing 3 times in aerated, dechlorinated tap water, eggs were incubated at room temperature for at least 28 days. Single worms of 5 age groups were similarly isolated to determine changes in fecundity over their life span of 225 days in mice.

Snails were maintained in 40-liter aerated aquaria and fed lettuce. Numerous offspring were thus produced for experimental infection. Exposed snails were kept in 11-cm fingerbowls at room temperature and fed lettuce. Water was added or replaced as required, usually weekly.

Snails were exposed singly or in groups of 2 to 5 to 1 to 10 miracidia/snail in small glass vessels. Other snails were exposed to *Z. lunata* eggs that had been incubated for 28 to 48 days, among which some miracidia appeared ready to hatch. Exposure to eggs continued for at least a week or more, since both snails and eggs were transferred to fingerbowls to be maintained until patency was observed.

To determine miracidial longevity, incubated eggs containing motile miracidia were illuminated with a 60-W incandescent lamp for 1 hr. All hatched miracidia were then transferred to a depression dish and observed microscopically until no motility was seen. The number of motile miracidia was recorded hourly.

Results

Infected *Helisoma anceps* (either field- or laboratory-infected) usually survived for more than 4 mo, providing a constant source of encysted metacercariae to infect mice. Mice necropsied after 35 days postinfection (PI) provided gravid adult worms from which large numbers of eggs were emitted. Eggs incubated at room temperature developed and began hatching on day 26, and continued to hatch until day 48. Hatching rates increased to a peak on days 35 to 40, then

Table 1. Oviposition by *Zygocotyle lunata* maintained at 37°C for 4 hr.

Worm age (days PI)	Number	Mean number of eggs/worm (±1 SD)
15–35	10	24 (±5.2)
36–70	10	76 (±11.1)
71–105	10	122 (±14.8)
106–140	10	84 (±12.4)
141–225	10	55 (±29.8)

* Worms older than 100 days produced more defective eggs and showed progressive gonadal hypertrophy as age increased.

declined gradually until day 48, after which no further hatching was observed. Based on counts of unhatched eggs in 10 dishes incubated for 50 days, hatching rates varied from 0 to 80%, usually about 50%. Among these groups of eggs, it was noted that eggs from adults maintained at room temperature had higher hatching rates and fewer abnormalities than eggs from worms kept at 37°C during oviposition. The rate of oviposition was dependent upon age of adult worms; maximum productivity was observed in 75- to 105-day-old worms (Table 1).

To test posthatching survival of miracidia, fully incubated eggs were allowed to hatch for 1 hr, then isolated and observed hourly. By 4 hr, more than 50% were dead (nonmotile), and none survived more than 8 hr (Table 2).

A series of 24 exposures of laboratory-reared *H. anceps* was performed, using from 1 to 10 newly hatched miracidia/snail. Snails ranged from 4 to 12 mm in diameter, and were exposed in groups of 1 to 5 in depression dishes with 2 ml of water. Miracidial penetration was rarely seen, but no miracidia were present after 4 to 5 hr. Exposed snails were observed for cercarial emergence for 100 days or more, unless death occurred. Dissections of all snails, living, moribund, or dead, revealed a single infection of daughter rediae on day 26 PI in a total of 56 exposed snails.

Table 3. Infection of *Helisoma anceps* exposed en masse to embryonated eggs of *Zygocotyle lunata*.

Group	Number and size of snails	Pre-patent mortality	Number infected (%)
A	15 adults (10–13 mm)	0	2/13%
B	4 adults (10–12 mm)	2	1/50%
C	8 adults (10–12 mm)	2	1/17%
D	3 adults (10–11 mm)	0	2/67%
E	6 adults (10–15 mm)	1	1/20%
F	13 adults (10–12 mm)	7	1/16%
G	55 young (2–3 mm)	0	9/17%
H	24 young (3–5 mm)	3	4/19%
I	12 young (3–6 mm)	4	2/25%
J	10 young (4–8 mm)	4	2/33%

Table 3 shows the results of exposing groups of snails to fully embryonated eggs. Ingestion of eggs was verified by observation of feces in which both hatched and unhatched eggs were seen. Some unhatched eggs contained partly embryonated, motile miracidia. At least 1 snail in each of the 10 exposed groups became patent using this exposure method. Cercarial emergence began as early as day 46 PI, but more often occurred after day 50; in 1 case, patency was not reached until day 84 PI. Infection rates are based on the number of patent snails as a percentage of surviving snails in each group.

Discussion

Few digenean parasites are routinely maintained and studied in all developmental stages. Consequently, the present state of knowledge of this large and diverse group of helminths is based on only a few species, usually on only those stages

Table 2. Longevity of *Zygocotyle lunata* miracidia.

Group	Number	Posthatching survival (hr)							
		1	2	3	4	5	6	7	8
A	6	5	3	3	1	1	1	0	—
B	10	8	8	5	4	2	1	1	0
C	12	11	9	6	5	3	0	—	—
D	9	9	7	5	4	3	2	1	0
E	15	12	9	9	8	4	1	0	—
F	18	14	12	12	9	7	5	1	1
G	7	6	5	3	3	1	0	—	—
H	14	13	10	7	6	4	1	1	0
Survival (%)	91	79/87%	65/71%	53/58%	44/49%	30/33%	17/19%	4/4%	1/1%

convenient to obtain, or having medical or veterinary importance.

Having identified a wild population of *Helisoma anceps* infected with *Zygocotyle lunata,* initial attempts to infect laboratory mice were highly successful, as were efforts to obtain and incubate eggs from gravid adults (Table 1). However, attempts to infect snails with hatched miracidia largely failed. Willey (1941) described methods of snail infection with *Z. lunata* and emphasized that miracidia hatched from incubated eggs "easily infected" helisome snails, a view perpetuated by Sey (1991). An alternative method of snail exposure described by Willey involved embryonated eggs. When I used this approach, a fairly high degree of success was obtained (Table 3). The explanation for poor results with hatched miracidia in the present study is probably due to the relatively short survival time of hatched *Z. lunata* miracidia (Table 2). Conversely, embryonation time for these eggs varies greatly, so that hatching occurs over a period of nearly 3 weeks (days 26 to 48 PI). This allows for a prolonged period of exposure when snails are placed in contact with unhatched embryonated eggs, most probably when snails ingest them. This inference is based on the dramatic difference in snail infection rates obtained with the 2 methods used in the present study. Regrettably, this method makes it difficult to measure the exact time required for snails to reach patency.

Optimum results in embryonating *Z. lunata* eggs were obtained by using adults 3 to 4 mo old (Table 1). While keeping adults at 37°C stimulates more rapid and copious egg production, it was noted that eggs obtained at this temperature were often abnormal in appearance and had lower hatching rates compared with eggs obtained at room temperature.

With the information gained in this study, it is now possible to begin critical experiments on various aspects of the growth, maturation, and longevity of all stages in the development of *Z. lunata* in both the snail and mammalian host, and to investigate further the relationships between this parasite and its hosts.

Acknowledgments

Appreciation is expressed to Mr. Wallace R. Horton for his permission to collect snails on his property, and to Dr. John B. Burch for confirming the identity of snails used in this study.

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